# Cloning, expression and purification of *Clostridium botulinum* neurotoxin type E binding domain

**Mir Latif Mousavi[1], Shideh Montaser Kouhsari[2], Shahram Nazarian[1], Iraj Rasooli[3] and Jafar Amani[1]**

*[1]Department of Biology, Faculty of Science, Imam Hossain University, Tehran. [2]Department of Biology, Faculty of Science, Tehran University, Tehran. [3]Department of Biology, College of Basic Science, Shahed University, Tehran, I.R. Iran.*

***Abstract***
*Botulinum neurotoxins constitute a family of bacterial toxins for botulism syndrome in human. The toxins bind with high affinity to nerve cells where they cause a complete inhibition and release of neurotransmitters and thereby produce flaccid paralysis. In this study the binding domain of type E neurotoxin was isolated by PCR and expressed in a proper expression vector. The results of this investigation can be used as a tool to study the mechanism of binding of holotoxins. This study is also implicated in antibody production against botulism syndrome.*
***Keywords:*** *Botulinum neurotoxin type E, Binding domain, Expression*

## INTRODUCTION

The botulinum neurotoxins (BoNTs) are the causative agents of botulism and represent a family of seven structurally similar but antigenically distinct serotypes (A to G) (Eric and Marite, 2001; Humeau *et al.,* 2000; Pellizzari *et al.,* 1999 and Chaddock *et al.,* 2002). These toxins exert their action by blocking the release of the neurotransmitter acetylcholine at the neuromuscular junction (Lalli *et al.,* 2003 and Turton *et al.,* 2002). BoNTs are usually expressed in *Clostridium botulinum* as a single polypeptide chain and then post-translationally nicked, forming a di-chain consisting of a 100 kDa heavy chain and a 50 kDa light chain held together by a single disulfide bond (Eric and Marite, 2001; Rossetto *et al.,* 2001; Zhou and Singh, 2004; Jensen *et al.,* 2003 and Agarwal *et al.,* 2004). Topologically, these neurotoxins are composed of three domains, a binding domain, a translocation domain, and a catalytic domain, each of which is believed to play a role in intoxication (Lalli *et al.*, 2003; Lalli *et al*., 1999; Zhou and Singh, 2004 and Jensen *et al.,* 2003). The carboxyl-terminal portion of the heavy chain is responsible for binding to nerve cell receptor(s). After toxin binding, it is thought to be internalized into an endosome through receptor-mediated endocytosis. It is believed that the 50 kDa amino-terminal domain of the heavy chain possesses channel-forming capabilities in the acidic environment of the endosome, allowing internalization of the toxin. The final step in the mechanism involves zinc-dependent proteolysis by the catalytic domain of key cytosolic substrates necessary for neurotransmitter release (Humeau *et al.,* 2000; Pellizzari *et al.,* 1999 and Turton *et al.,* 2002). The *Clostridium botulinum* neurotoxins specifically cleave a family of proteins, named SNAREs (soluble NSF attachment protein receptor) (Kotich *et al.,* 2002 and Rickman *et al.,* 2004). These proteins are involved in multiple steps leading to the docking and fusion of small synaptic vesicles with the presynaptic plasma membrane and in a variety of other intracellular trafficking events. BoNT/E instead, cleaves Arg180-Ile181 peptide bond of the SNAP-25 (synaptosomal-associated protein of 25 kDa) (Schiavo *et al.,* 1998 and Pellizzari *et al.,* 1999). The binding domain of BoNTs has been one of the most successful and frequently used tools in neurobiology and cell biology. Recombinant heavy chain C-terminal (HCc) fragment of BoNT/E antagonised the action of parental *Clostridium botulinum* neurotoxins, which is seen as a delay in the onset of paralysis (Lalli *et al.,*

*Correspondence to:* **Mir Latif Mousavi, PhD**
*Tel: +98 21 7313934, Fax: +98 21 7313934*
*E-mail: mmousavi@ihu. ac. ir*

1999). A survey of bottom sediments of the Caspian Sea prompted by a relatively high level incidence of botulism associated with consumption of fish. Type E spores were most prevalent (%92), while type B spores were also present (%8). In new search, of 34 fresh fish from the Caspian Sea, 12 (%35) were positive with type E (Cato and Stackebrandt, 1989). On the other hand, difficulties were encountered in production of toxoid vaccines (Smith. 1998; Byrne and Smith, 2000). Draw backs such as spore formation by *C. botulinum*, low yields and handling risk of toxin, high cost of manufacturing, reactogenic nature of toxoid imparted by the use of formaldehyde and other chemicals such as thimerosal, prompted us to think of producing a recombinant protein. Expression of BoNT/E in a suitable expression vector will facilitate antibody production applicable for protection against botulism syndrome. This could result in easy production of a cheaper and safer immunogen.

## MATERIALS AND METHODS

**Enzyme and chemicals:** T4 DNA ligase and *Pfu* DNA polymerase were from Fermentase. All other chemical reagents were obtained from Sigma (USA) and LaRoche (Germany). Miniprep kit for purification of DNA was from Qiagen (USA). Primers were synthesis by MWG Biotech Company (Germany). Specific antibody against *Clostridium botulinum* type E neurotoxin was the product of Medep Co. (Russia).

**Bacterial strain and plasmids**: Plasmid pUC18 was purchased from Pharmacia and PET32a was the product of Novagen (USA). The Plasmid pTZ57R obtained from Fermentase. *Escherichia coli* BL21 DE3 and DH5α were from Cinagene (Iran).

**Microbial cultures**: *E. coli* strain was grows in LB broth and *Clostridium botulinum* type E was grown in cooked meat (Difco, USA).

**Bacterial growth and isolation of DNA:** *Clostridium botulinum* type E cells were cultured anaerobically under 15% $CO_2$ at 35°C for 24-48 h in cooked meat medium Chromosomal DNA was prepared with alkaline method.

**PCR amplification and cloning:** A pair of primer 5′ATAC GGATTC AAT AAT AGT ATT CCT TTT AAG C 3′ which adds a *Bam*H1 site to the 5′ end and 5′ TCTA GTCGAC TTA TTT TTC TTG CCA TCC 3′ which adds a *Sal*1 restriction site to the 5′ end were designed from the published binding domain of BoNT/E (accession # x62683) to isolate this domain as residue 830-1251. PCR was performed in 50 µl total volume containing *Pfu* DNA polymerase (2 units) and its buffer, 0.2 mM of each dNTP, 20 pmol of both primers, and 20 µg DNA. Samples were preheated for 5 min at 95°C and then 30 cycles of PCR were performed: 1 min at 94°C, 30 s at 60°C, and 1.5 min at 74°C. After the last cycle, was continued for additional time of 7 min at 74°C. The size of amplified DNA fragment as monitored on 1. 0% agarose gel was ~1.3 kb. The PCR product was isolated from a low melting agarose gel and purified with gel extraction kit and digested with *Bam*H1 and *Sal*I. The digested PCR product was ligated into a pTZ57R previously digested with the same enzyme and transferred into DH5α host. One recombinant plasmid containing the expected size insert was sequenced by the dideoxy chain termination method. The recombinant plasmid was cleaved with *Sal*I and *Bam*HI, the *Sal*I–*Bam*HI fragment was isolated and recovered from the low melting agarose gel, and then ligated to the expression vector pET-32a. Clones were screened by digestion of a miniprep plasmid DNA with *Sal*I and *Bam*HI.

**Expression and Purification:** Five milliliters of LB Broth medium containing 50 mg/ml ampicilin was inoculated with 10 µl of the freezer stock of BL21 (DE3) cells with pET-BoNT/E binding domain. Following an overnight growth at 37°C, this culture was used to inoculate 100 ml of LB ampicilin medium in a 500 ml flask and the cells were grown at 37°C with shaking until the $A_{600}$ reached 0.6. At this point, 1mM IPTG was added to induce BoNT/E-HCc expression. The cells were then incubated at 30°C for an additional 12h. The induced cells were harvested by centrifugation at 5000 rpm at 4°C for 10 min. The pellet was resuspended in 4 ml lysis buffer (50 mM Na–phosphate, pH 8. 0, 300 mM NaCl, 5 mM benzamidine and 0. 5 mM PMSF) supplemented with protease inhibitor and lysozyme. The bacterial suspension was incubated at room temperature for 30 min to lyse cells. After the cells were completely lysed, 5 µg/ml deoxyribonuclease- I was added to digest any associated DNA. This step helps reducing the viscosity of the extract. After an additional 10 min, the lysate was centrifuged at 16000 rpm for 30 min to remove the insoluble cell debris. Each of the culture samples was tested for the expression of protein on sodium dodecyl sulfate–polyacrylamide gel (10%) electrophoresis (SDS–PAGE). The supernatant obtained from the above step was allowed to mix with 5 ml Ni–NTA

agarose (nickel-nitrilotriacetic acid), pre-equilibrated with phosphate buffer (50 mM Na–phosphate, pH 8.0, 300 mM NaCl), at 4°C for an hour. The mixture of Ni–NTA agarose and supernatant was poured into the glass column and the flow-through of the soluble fraction was collected. The column was washed with 10 ml of phosphate buffer followed by 5 washes with buffer containing 50 mM immidazole. Tightly bound protein was eluted with 2 ml of 100 mM and 3 ml of 200 mM immidazole. Aliquots of all the above fractions were analyzed by electrophoresis on 12% SDS–PAGE gel followed by staining with Coomassie blue. A 71 kDa band corresponding to BoNT/E-HCc reproducibly eluted in 100–200 mM immidazole fractions was obtained. In order to separate the fusion protein from HCc chain the recombinant protein was treated with restriction protease enterokinase. The reaction was carried out as described in the manufacturer's (Roche, Germany) manual. The protein was further purified employing Ni–NTA agarose column.

**Western blotting analysis:** The recombinant protein was detected by Western blotting using horse anti-*clostridium botulinum* toxin type E. The recombinant BoNT/E HCc was transferred to nitrocellulose membrane using Bio-Rad Mini Protean II System and transfer buffer (39 mM glycine, 48 mM Tris–base, 0.037% SDS, and 20% methanol). The membrane was incubated in the blocking buffer of 3% bovine serum albumin (BSA)/ phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, and 4.3 mM $Na_2HPO_4 7\ H_2O$, pH 7.3), with gentle shaking for 1 h at room temperature. After decanting and discarding the blocking buffer, the membrane was incubated in a 1:3000 dilution of horse anti-*clostridium botulinum* toxin type E in the PBST (PBS containing 0.05% Tween), with gentle shaking for 1h at RT. After washing the membrane with PBST for three times, each time for 5 min, blots incubated with a 1:1000 dilution of the polyclonal goat anti-horse HRP conjugate. The blot was washed three times in PBST and stained with HRP staining solution (DAB). Chromogenic reaction was stopped by rinsing the membrane twice with water.

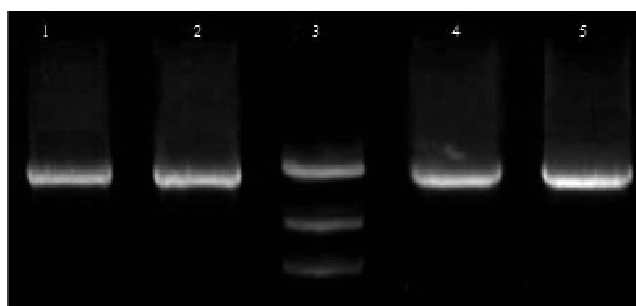


**Figure 1.** PCR product of Clostridium botulinum type E Lane 3: Puc18/Taq1 marker; lanes 1, 2, 4, 5: BonT/E binding domain gene (1302 bp).

## RESULTS

A DNA fragment encoding the binding domain of BoNT/E was amplified by PCR using sense and antisense primers. Specific restriction sites, *Bam*HI and *Sal*I, for unidirectional cloning and a stop codon were introduced into the amplified gene. *Pfu* DNA polymerase with proofreading was used for amplified gene (Fig. 1). The restriction map of the binding domain gene with *Eco*RI and *Bcl*I derived by software analysis showed three fragments of 811, 385 and 187 and two fragments of 978 and 324 base pairs which were produced on digestion with *Eco*RI and *Bcl*I respectively. The amplified fragment was thereby confirmed to be the correct one. The isolated DNA from low melting agarose was ligated into *Bam*HI, *Sal*I-cleaved pTZ57R vector. Restriction digestion and subsequent agarose gel electrophoresis of the clone indicated the presence of a 1302 bp fragment (Fig. 2). In an attempt

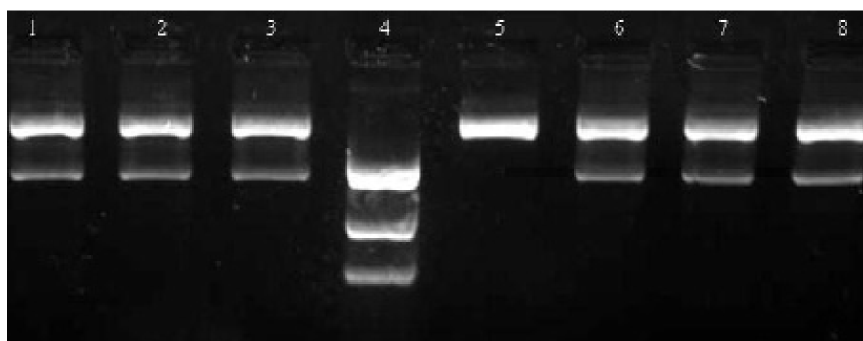


**Figure 2**. Digestion products of pTZ57R with *Bam*HI and *Sal*I; lane 5 pTZ57R without insert; lanes 1, 2, 3, 6, 7, 8 clones; Lane 4: pUC18/Taq1 marker.

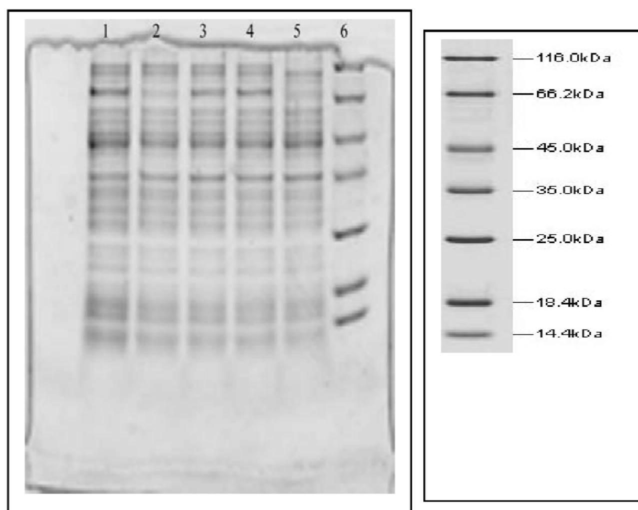


**Figure 3.** SDS-PAGE analysis of total cell lysate of *E. coli* BL21 DE3 containing pET32a showing the expression of binding domain at different colonies (lanes 1-4). Lysate samples were prepared from 1 ml of cell suspension induced with IPTG. The cells was harvested and resuspended in 100 µl 2x SDS gel loading buffer, heated to 100°C for 4 min, and loaded onto 10% polyacrylamide gel. Lanes 1-4, cell lysate of *E. coli* BL21 DE3 containing pET32a after induction with IPTG. Lane 5, before induction with IPTG. Lane 6, protein marker.

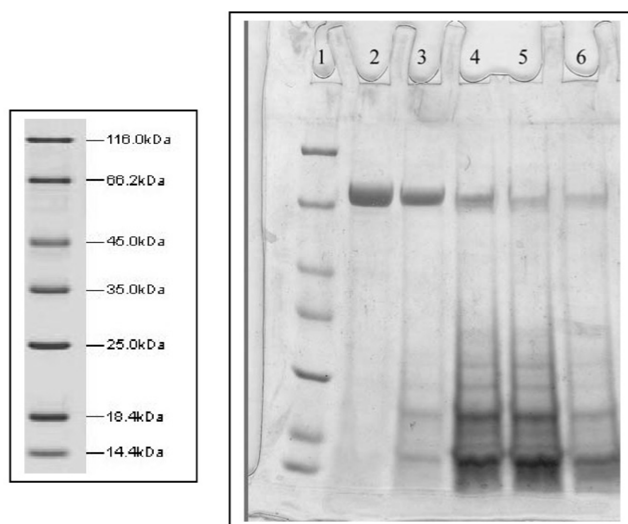


**Figure 4.** Purification of the recombinant binding domain (pET32a-HCc) on Ni-NTA affinity column. Proteins were separated on a 10% SDS-PAGE gel and Commassie blue stained. Lane 6, 20 mM imidazole wash fraction. Lane 5, 50 mM imidazole eluate fraction. lane 4,100 mM imidazole eluate fraction. Lane 3, 150 mM imidazole eluate. Lane 2, 200 mM imidazole eluate. Lane 1, protein marker.

to get a higher yield, the DNA fragment cloned into pTZ57R, was excised with *Sal*I and *Bam*HI, and then ligated into pET-32a which is a bacteriophage T7-RNA polymerase promoter vector system. The T7-RNA polymerase is capable of transcribing almost any DNA linked to a T7 promoter (Studier *et al.,* 1990). The pET-BoNT/E-HCc recombinant plasmid was digested with *Nde*I and *Bam*HI or *Sal*I, or *Bam*HI only, to confirm the ligation. The recombinant plasmids were also confirmed by PCR. DNA sequence analysis confirmed its correct orientation when compared to the sequence of binding domain. The pET-BoNT/E-HCc was transformed into *E. coli* BL21. His6-tagged recombinant BoNT/E-HCc was found to be overproduced using IPTG as an inducer. A band corresponding to a 71 kDa His 6-tagged BoNT/E-HCc was observed in SDS–PAGE of total lysate of *E. coli* BL21 (pET-BoNT/E-HCc) culture after induction with IPTG (Fig. 3). The soluble fraction of the protein was allowed to selectively bind to Ni–NTA agarose through the His-tag. The protein was then eluted by ascending concentrations of immidazole. The protein was eluted largely at 200 mM immidazole concentrations (Fig. 4). The 71 kDa purified recombinant protein was digested with enterokinase as a result of which a 50 kDa HCc and 20 kDa fusion protein could be identified (Fig. 5). The protein identity was verified by Western blot analysis (Fig. 6). The assay revealed that the recombinant BoNT/E-HCc was strongly and specifically recognized by anti-*clostridium botulinum* toxin type E., Whereas, no reactivity was observed in controls.

## DISCUSSION

The *Clostridium botulinum* toxins (type A-G) consist of two protein chains joined by a disulphide bridge. The light chain (50 kDa) is responsible for the intracellular catalytic (toxic) activity and heavy chain (100 kDa) for the binding and internalization of the toxin into the neuron. In particular it is the 50 kDa C-terminal domain of the heavy chain which is responsible for the binding of the toxin to neuronal membrane prior to toxin internalization. Recombinant protein of this construct has potent antagonists of the corresponding clostridium neurotoxins (Lalli *et al.,* 1999), causing a significant delay in the onset of paralysis more than 150%, while percent for binding domain of BoNT/A and TeNT is lower than 75%. Therefore the data obtained indicates that the binding domain can be cloned and expressed in *E. coli* and recombinant protein can be considered for production of antibody. Recent works (Smith, 1998 and Byrne and Smith, 2000) have also shown that binding domain gene derived from botulinum toxins A and B can be exploited as vaccines against their respective toxin subtype.

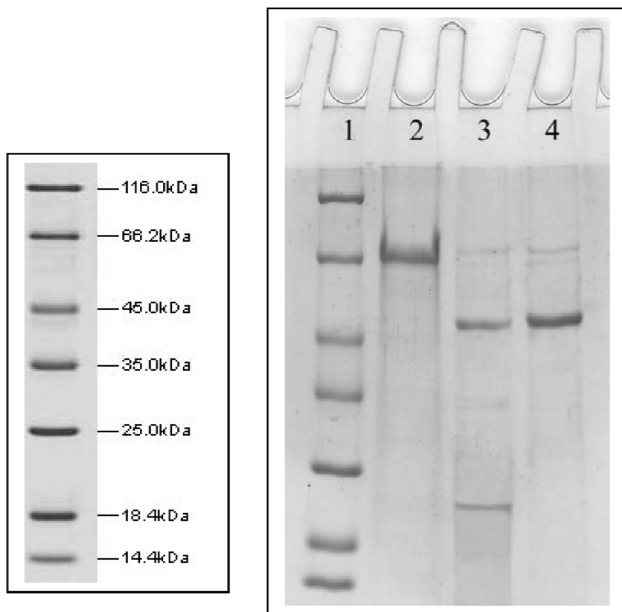


**Figure 5.** Digestion 71 kDa recombinant protein with enterokinase. Lane 1, protein molecular weight marker. Lane 2, 200 mM washed fraction from Ni–NTA agarose affinity column. Lane 3, enterokinase digestion product (~ 50 kDa HCc and 20 kDa fusion protein). Lane 4, recombinant binding domain without fusion.

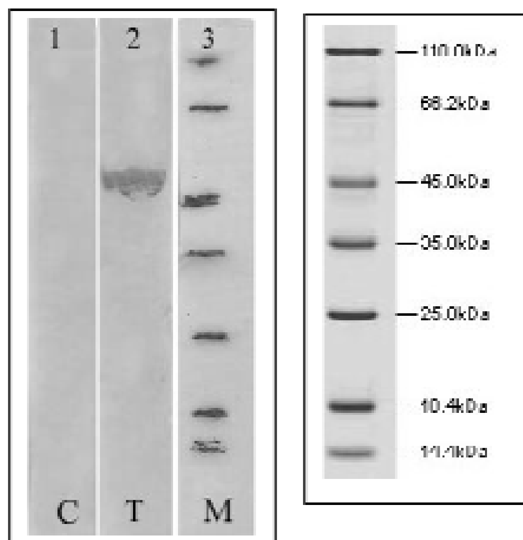


**Figure 6.** Western blot of the binding domain; Lane 1, supernatant from *E. coli* clone without insert. Lane 2, Binding domain protein without fusion. Lane 3, Protein Molecular Weight Marker.

On the basis of the structural similarity between these toxins and botulinum toxin E we investigated the possibility of exploiting carboxy-terminal fragment from the heavy chain of botulinum E toxin as vaccine. Although toxoid vaccines are available, there are numerous shortcomings with the current use and ease of production. Development of a new generation, recombinant vaccine could alleviate many of the problems associated with the toxoid. A recombinant vaccine would eliminate the need for a dedicated manufacturing facility. Presently, many current Good Manufacturing Practices (cGMP) facilities are in existence and available that could manufacture a recombinant product. There would be no need to culture large quantities of a hazardous toxin producing bacterium. Production yields from a genetically engineered product are expected to be high. Presumably, there would be no need to treat the vaccine with formalin if the recombinant vaccine candidate represented a fragment of the toxin. A fragment would not possess all three functional domains (i.e., binding, internalization, catalytic) which are all required for its mechanism of action. Recombinant products would be purer, less reactogenic, and more fully characterized. Human immunization with toxoid resulted in production of antibodies directed largely against the toxin light chain, with fewer antibodies binding heavy chain. Since antibody neutralization activity results largely from blockade of cellular receptor binding by heavy chain, so the heavy chain vaccine will be more protective than a toxin based vaccine, as more heavy chain antibodies are generated (Amersdorfer *et al.,* 2002). Thus, the cost of a recombinant product would be expected to be much lower than a toxoid because there would be no expenditures required to support a dedicated facility, and the higher production yields would reduce the cost of the vaccine product.

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